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THE TOXICOLOGY OF RICIN AND ABRIN TOXINS - STUDIES ON IMMUNISATION AGAINST ABRIN TOXICITY

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ABSTRACT

The toxicology of ricin and abrin toxins has been examined in rats by head only exposure to a range of concentrations, generated from solution as aerosols of mmad 0.9 μm. The approximate LCt_{50}s were determined for each toxin and found to be very similar (LCt_{50} ricin 4.54-5.96, abrin 4.54 $mg.min.m^{-3}$). In both cases pathology was found to be restricted to the lung and included necrosis of lower airway epithelium, degeneration of type I pneumocytes accompanied by severe interstitial and intra-alveolar oedema; this feature, accompanied by hypoxia, was the cause of death. The recovery phase was typified by hyperplasia of type II epithelial cells (particularly florid for abrin) and gradual disappearance of protein-rich alveolar oedema fluid.
From studies performed in vitro using bovine pulmonary endothelial cells, inhibition of protein synthesis occurred from 30 minutes incubation with abrin or ricin (LC_{70} concentrations) while decrease in viability did not occur until between 15 and 24 hours under the same conditions. Given this information, the time frame for therapeutic intervention is likely to be small and therefore immunisation would appear to be the best option for protection against abrin or ricin toxicity. We have examined the efficacy of immunisation with abrin toxoid in the rat in terms of immune-status and effect of subsequent challenge with multiple LCt_{50}s of the native toxin.

INTRODUCTION

Ricin is a toxic protein of molecular weight 65 kDa extracted from the seeds of the Castor oil plant, *Ricinus communis*. The structure and function of ricin, abrin and other similar plant toxins have been well examined and reviewed [1]. Ricin is composed of two dissimilar glycoprotein chains linked together via a single disulphide bond. The larger B chain (34 kDa) binds the whole toxin molecule to galactose residues in oligosaccharide sequences on lipids or proteins present in membranes of cells. B chain assists internalisation of the smaller A chain (32 kDa) which inhibits protein synthesis in eukaryotes enzymically, inactivating the 60S ribosomal subunit. It has been shown [2] that ricin A chain modifies both or either G_{4323} and A_{4324} nucleoside residues in 28S rRNA and that other toxins including abrin and modeccin, exhibit similar activity. It was later confirmed [3] that ricin A chain behaved as an N-glycosidase cleaving this linkage between the base and ribose at position A_{4324} in the rRNA.

Abrin is a toxic protein prepared from the seeds of the Jequirity plant (*Abrus precatorius*) and which shares structural and functional principles with ricin. Comparison of minimal lethal doses when administered intravenously to mice [4] shows abrin (0.7 µg/kg) to be approximately four times as potent as ricin (2.7 µg/kg).

Endothelial cells were chosen as a model for the toxicity of ricin and abrin as earlier observations in vivo [5] indicated indirectly, that these toxins have damaging effects on capillary endothelia. Haemorrhagic lesions were evident macroscopically in bowel, lymph nodes and gut-associated lymphoid tissue (GALT) of rats injected intramuscularly with either toxin. Microscopic examination of lymph nodes from poisoned rats demonstrated the presence of erythrocytes in the sinuses.

In order to develop new pretreatments and therapies this study has investigated the toxicology and mechanisms of action at a cellular level.

METHODS

Toxin preparations

The initial inhalation toxicity and pathology of ricin were performed on toxin purchased from Sigma Chemical Company Ltd, Dorset, UK. Subsequent studies in vitro and in vivo were performed on toxins prepared 'in house', from seeds of *Ricinus communis* or *Abrus precatorius*.

Both toxins were purified by a method based upon that for the isolation of *Viscum album* lectin, using affinity chromatography on Sepharose 4B [6]. In our method, in order to separate toxic lectins from the less toxic, larger agglutinin molecules, a further chromatography stage on Superdex 200 was incorporated and all protein separations were performed using FPLC.

Aerosol generation and exposure of experimental animals to ricin and abrin.

All procedures were performed in modified class III (BS 5726) microbiological containment cabinets made of stainless steel; each component cabinet had its own filtered air inlet and exhaust system.

The mass median aerodynamic diameter (MMAD) of the dried aerosol was calculated to be 0.89-0.91 µm (σg = 1.6). Challenge Ct's were adjusted between groups by changing either the airflow, and hence the aerosol concentration, or the exposure period. Groups of six animals were exposed, head only, to a single dose of the toxin aerosol.

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Histopathological examination

In the ricin study, selected animals from the entire exposure dose range (Ct 119.3 - 1.51 $mg.min.m^{-3}$) were humanely killed at 1 to 21 days post-exposure for histopathological examination (see Table 1). Animals which died during the working day were also examined histopathologically.

For abrin, a separate group of 24 rats was exposed to an approximate LCt_{50} (3.4 $mg.min.m^{-3}$) of abrin and animals were humanely killed in groups of 3 at 2, 3, 4, 7 and 14 days post-exposure. All major tissues were assessed by routine histopathology.

Immunisation

Rats were immunised subcutaneously with three injections of formaldehyde-toxoided abrin (125 $\mu g.kg.^{-1}$ bodyweight), separated by a time interval of three weeks. Samples of blood, to provide serum for analysis, were taken (tail vein) immediately before each injection and finally, before challenge with aerosolised abrin toxin (approximately 5 x LD_{50}). Animals were observed over a period of 14 days after challenge. Titres of anti-abrin antibodies and examination of the main antibody classes present in sera and bronchioalveolar lavage fluid were measured by ELISA. Pathological examination of rats exposed to abrin or ricin was carried out.

Cell Culture: Bovine pulmonary endothelial cells

Bovine Pulmonary Endothelial (BPE) cells of aortic origin were grown in DMEM containing FCS (referred to as full DMEM).

Exposure of BPE cells to toxin

The BPE cells were exposed to toxin after passaging the cells and seeding them into flat-bottom 96-well cell culture plates. After various incubation times (at 37°C) in a humidifed atmosphere of 5% CO_2/95% air, the effects of toxin were determined.

Unless otherwise stated, all other operations were carried out at room temperature. Viability under experimental conditions was related to that of control cells in full DMEM only (the reference 100% viable cell control).

Measurement of viability

Cell viability was determined by the neutral red (NR) assay [7], MTT [8] and gentian violet (GV) [9] assay systems.

Toxicity of abrin and ricin

BPE cells were sown into 96-well plates as previously described. Medium containing 0 to 500 pM toxin was added to the wells and the cells incubated for 24 hours prior to their viability being assessed by the NR assay.

The use of BPE cells and the GV, MTT and NR assay systems to determine the time course of events following exposure to abrin and ricin

BPE cells in 96-well plates were incubated in medium only or with medium containing an LC_{70} dose of abrin and ricin. The cells were pulsed with the toxins for 3 to 48 hours. Cell viability was determined at the end of each time point using the GV, MTT and NR assay systems.

Exposure of BPE cells to LC_{70} and 5 x LC_{70} concentrations of abrin and ricin over time using the NR assay

In this experiment BPE cells were incubated for 24h (96-well plates) after pulsing with medium only or medium containing LC_{70} and 5 x LC_{70} concentrations of toxin from 2.5 min to 24h. Viability was assessed at 24h by the NR assay.

Time-course of inhibition of protein synthesis by abrin and ricin toxins

BPE cells were grown to confluence, as described above, in flasks (25 cm^2) and LC_{70} doses of abrin or ricin were added for time periods of 5, 10, 20 and 30 minutes, 1.5, 2, 3, 4 and 5 hours. For each time period examined 6 replicate flasks were set up. After incubation with toxin, medium was replaced with fresh full DMEM containing ^{3}H-leucine and incubated further for 90 minutes as above. Triton X-100 (10% v/v) was then added to the contents of each flask. Trichloroacetic acid (TCA) was added and the contents were mixed and the flasks left overnight. 24 hours later, flask contents were suspended by shaking and centrifuged. Pellets were resuspended in 10% TCA and recentrifuged. The pellets were finally resuspended in 0.1 M NaOH. Counts incorporated into protein were determined and related to DNA content [10]. Protein synthesis for intoxicated cells was expressed relative to that of control cells as % inhibition for each toxin pulse time.

RESULTS

Inhalation toxicology of ricin and abrin toxins

The results of inhalation of ricin and abrin are summarised in Tables 1 and 2. For ricin, at the higher CT's of 119.3 and 11.9 $mg.min.m^{-3}$, animals were humanely killed at time points shorter than the time to death of their companions. These exhibited severe clinical signs of poisoning from which recovery was considered to be unlikely. At the lower Ct of 5.96 $mg.min.m^{-3}$, four animals died at between 2.75 and 3.5 days; the surviving two animals were examined at 5 days, when histopathology showed recovery to be well under way. At a Ct of 4.54 $mg.min.m^{-3}$, one animal in the group died and the remaining five survived to be humanely killed at 5, 7 and 14 days respectively. The LCt_{50} of ricin was assessed to be between 4.54 and 5.96 $mg.min.m^{-3}$ and the LD_{50} was approximately 3.7 $\mu g.kg^{-1}$. For abrin, the approximate LCt_{50} was 4.54 $mg.min.m^{-3}$ and the estimated LD_{50} was 3.31 $\mu g.kg^{-1}$. In general, survival time was inversely related to exposure dose of either toxin, with deaths in the highest Ct exposure groups occurring at between 36 and 42 hours post-exposure.

Histopathology of ricin and abrin toxins

The pathology resulting from inhalation of each toxin was similar and confined to the lung. Common features included bronchiolar epithelial damage featuring necrosis and apoptosis, intra-alveolar oedema, macrophage infiltration of the lung parenchyma and acute alveolitis. Tissue regeneration was typified by hyperplasia of type II pneumocytes. The time to onset of pathological features was later at lower Cts.

Differences in the pathological features between abrin and ricin were as follows:

1. There was no apparent damage to the epithelium of the upper respiratory tract (larynx and trachea) following abrin inhalation but this was observed after inhalation of ricin.
2. Apoptosis, an abundant feature in lung parenchyma after ricin intoxication was far less evident in animals which received abrin. Apototic bodies were present in the bronchiolar epithelium of rats after inhalation of either toxin.

TABLE 1 Toxicity of Ricin by Inhalation in the Rat

Exposure (minutes)	Aerosol conc ($\mu g.L^{-1}$)	Ct ($mg.min.m^{-3}$)	Inhaled Dose ($\mu g.kg^{-1}$)	Mortality (Number)	Autopsy (day)
40	2.98	119.3	85.77	a (5/5)	1
4	2.98	11.93	7.67	b (6/6)	1,2 &3
2	2.98	5.96	3.83	c (4/6)	5
3	1.48	4.54	3.55	(1/6)	14 & 21
2	1.48	3.01	2.02	Nil	5,7 & 2
2	0.57	1.51	1.05	Nil	5,7 & 1

a One animal died at 1.25 days, three at 1.50 days, one moribund humanely killed at 1 day.
b One animal died at 3 days, one at 3.75 days, one at 5.5 days, three chosen at random for autopsy.
c Two animals died at 2.75 days, two at 3.50 days, two survivors humanely killed at 5 days.

TABLE 2 Toxicity of Abrin by Inhalation in the Rat

Exposure (minutes)	Aerosol conc ($\mu g.L^{-1}$)	Ct ($mg.min.m^{-3}$)	Inhaled Dose ($\mu g.kg^{-1}$)	Mortality (Number)
16	1.42	22.7	16.7	a 6/6
16	0.56	9.1	6.62	b 6/6
8	0.56	4.54	3.31	c 3/6
8	0.14	1.14	0.83	Nil
4	0.14	0.57	0.43	Nil
2	0.14	0.28	0.20	Nil

a All six rats died at approximately 1.75 days post-exposure
b Two rats died at 1.75, two at 2.5 and two at 2.75 days post-exposure
c Deaths occurred at 2.75, 3.75 and 5.75 days post-exposure

As for ricin, time to death was generally inversely proportional to dose received

3. During the resolution phase, the regenerative hyperplasia of type II pneumocytes seen after both toxins was particularly marked for abrin 3-4 days after exposure.

Immunisation study

Anti-abrin antibody titres and major immunoglobin classes present were assayed by ELISA on the blood samples taken from all rats immunised with abrin toxoid (3 weeks after each of the 3 toxoid injections).

1. Average titres measured after the three injections were 1/1600 (1st), 1/12800 (2nd) and 1/25600 (3rd).
2. At the end of the immunisation period, inmmunoglobulins of types G and A were present in a ratio of approximately 80% : 20% respectively.
3. Ten rats were challenged to 5 LD_{50}s of abrin toxin by inhalation, 3 weeks after the third immunising boost of abrin toxoid. Six rats were observed for effects of toxin challenge over 14 days; all rats survived the lethal aerosol challenge.
4. Four rats were killed humanely 24 hours following challenge and bronchioalveolar lung fluids were extracted, and examined. Anti-abrin titres ranged from 1/256 to 1/1024. IgG and IgA at a ratio of approximately 70% : 30% respectively, were detected.

Time course of ricin and abrin toxicity in BPE cells

LC_{50} values of 1.9 and 5.1 pM were found for abrin and ricin respectively (Figure 1) using the NR assay. There were no differences in viability of the BPE cells (measured by the GV, MTT and MR assays) with time at the respective LC_{70}s of abrin and ricin (4.5 and 15.5 pM) (Figures 2a to 2c). Cell viability, as determined by the GV assay, was maintained at 70 to 100% for up to 2 hours before a more substantial decline in the viability of the cell population (Figure 2a). The MTT assay (Figure 2b) indicated a gradual decline in mitochondrial activity over the entire time period studied. When viability was assayed using NR (Figure 2c) there was little change between 3 to 20 hours after which point viability declined relatively suddenly. BPE cells were pulsed with LC_{70} and 5 x LC_{70} concentrations of abrin or ricin for 2.5 minutes to 24 hours (Figure 3). Cells pulsed for up to 45 minutes with an LC_{70} concentration of abrin or ricin produced no cell death at 24 hours (NR assay) though longer pulses of toxin gradually reduced cell viability, so that pulsing with either toxin over 24 hours reduced cell viability to 30% as expected. Pulsing with a 5 x LC_{70} dose resulted in a reduction in cell viability to approximately 26% after 24 hours with after a 2.5 minute exposure to either toxin.

Time-course of Inhibition of protein synthesis

Comparison of ^{3}H-leucine incorporation into cellular protein in the presence or absence of either toxin (LC_{70}) was used as a measure of the % inhibition of protein synthesis. There was no change in the rate of protein synthesis until toxins and cells had been incubated together for at least 30 minutes (ricin) or 1.5 hours (abrin) (Figure 4). After this time the rate declined steadily, and the slope of the inhibition line for abrin was steeper than that for ricin.

DISCUSSION

Abrin is several times more potent than ricin by systemic administration [4], but this study showed that both toxins were of similar potency when administered by the inhalation route. Ricin is therefore of comparable toxicity when administered by inhalation or parenteral routes [11] but abrin is less potent by inhalation. The main difference between the inhalation and other routes of administration is the distribution of the toxin and the subsequent pattern of pathology. By the intravenous and intramuscular routes, the toxin can be traced widely throughout the body [13-17]. This study suggests a much more restricted distribution with primary pathological changes confined to the respiratory system.

FIGURE 1. Viability of BPE cells after incubation with abrin and ricin.

Ricin and abrin toxins were each diluted in full DMEM over a range of concentrations including 0, 0.8, 4.0, 20, 100 & 500 pM. Plates were divided into blocks of 9 wells, each block being replicates of one datum point. Toxin solutions (0.1 ml volumes) were added to cells and incubated for 24 hours prior to conducting a viability assay. (Data points represent mean values, +/- standard deviation, n=9). Viability (absorbance at 540 nm) of test wells is expressed as a percentage of that of the control cells.

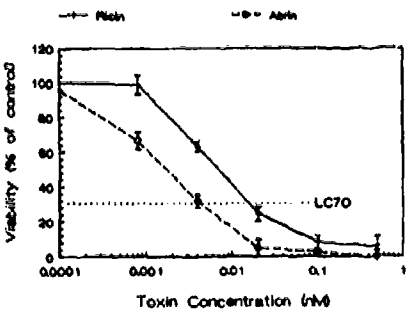
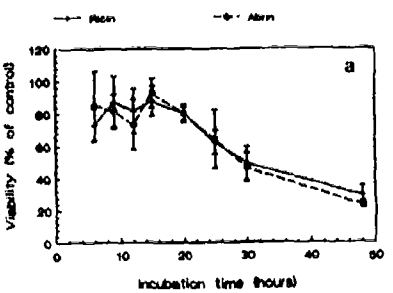
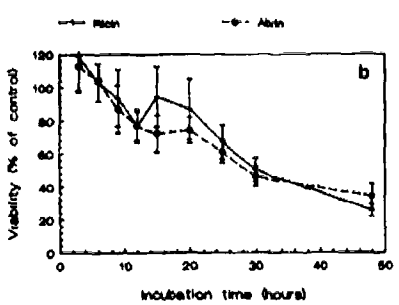


FIGURE 2. Effect of exposure of BPE cells to LC_{70} concentration of abrin and ricin over time using the GV, MTT and NR assays as indices of cell viability.

In this experiment BPE cells were incubated (96 well plates) with full DMEM only (control) or including ricin or abrin at LC_{70} concentrations over a 48 h time course. Viability was assessed at 3, 6, 9, 12, 15, 20, 25, 30 & 48 h after addition of toxin (or full DMEM only) by the GV (a), MTT (b) and NR (c) assays. (Data points represent mean values, +/- standard deviation, n=9).





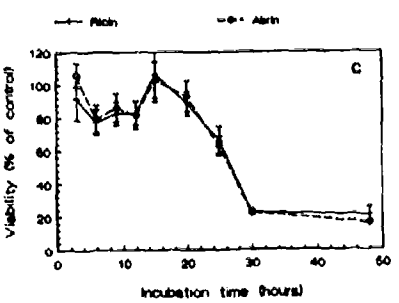
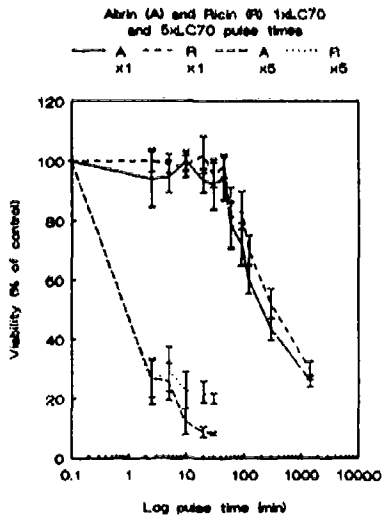


FIGURE 3. Effect of exposure of BPE cells to LC_{70} and 5 x LC_{70} concentrations of abrin and ricin over time using the NR assay as an index of cell viability.

In this experiment BPE cells were incubated for 24 h (96 well plates) after pulsing with DMEM or LC_{70} and 5xLC_{70} concentrations of toxin from 2.5 min to 24 h. Viability was assessed at 24 h by the NR assay. (Data points represent mean values, +/- standard deviation, n=9).



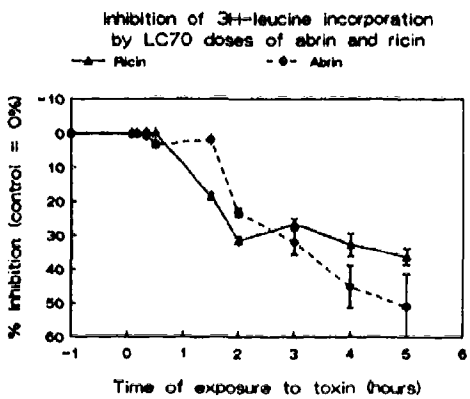


FIGURE 4. Inhibition of ^{3}H-leucine incorporation in BPE cells exposed to LC_{70} concentrations of abrin and ricin

BPE cells were grown to confluence in flasks ($25cm^2$) and LC_{70} doses of abrin or ricin were added for time periods of 5, 10, 20 and 30 minutes, 1.5, 2, 3, 4 and 5 hours. After incubation with toxin, medium was replaced with fresh full DMEM containing 0.37 $MBq.ml^{-1}$ of ^{3}H-leucine and incubated further for 90 minutes. 240 μl of Triton X-100 (10% v/v) was added to the contents of each flask followed by agitation. Trichloroacetic acid (4 ml; 20% w/v) was added and the contents were mixed and the flasks left overnight. 24 hours later, flask contents were suspended by shaking, and centrifuged at (4300 x g). Pellets were resuspended in 10% TCA and recentrifuged, resolubilised in 0.1M NaOH, and incorporated counts determined. The incorporated radioactivity was normalised to the number of counts μg^{-1} DNA. Protein synthesis for intoxicated cells was expressed relative to that of control cells as % inhibition for each toxin pulse time. (Data points represent means of six replicates +/- standard error).

The time to onset of pathology was delayed with lower dosages, but the pathology caused by both of the toxins was independent of dose. Deaths were apparently caused by hypoxia resulting from overwhelming oedema, which arose from direct damage to the respiratory epithelium and possibly also to the alveolar capillary endothelium. The target cell for ricin and abrin in the deep lung was the type I pneumocyte, which has cell surface receptors for Ricinus communis agglutinin (I) [12]. It is known that following damage to the type I cells, the type II cells proliferate and differentiate into type I cells [12], and this study confirmed both the initial damage to type I cells and the proliferation of type II cells. Ricin and abrin have both been associated with apoptosis in body tissues after intramuscular injection [17], however large numbers of apoptotic bodies were not seen here after inhalation of abrin. This is in contrast to the marked apoptosis seen in the rat lung after ricin inhalation. The reason for the absence of apoptosis following inhalation of abrin is unclear. It is apparent that in future studies 28 days should be allowed after challenge for complete resolution of any pathology, and the scope of the study should be extended to establish whether there are residual effects on lung function after the resolution of pathology which would limit exercise potential.

Immunisation by subcutaneous injection of abrin toxoid produced high titre antisera (IgG and IgA) and also protected all immunised animals against the lethal effects of abrin. However, immunisation did not completely prevent the development of pathology after challenge with abrin; these results agree with similar results in mice immunised with ricin toxoid [18].

Abrin was 2.7 times more toxic than ricin in the NR assay, reflecting their in vivo toxicities [5]. The no-effect concentration for abrin was 160 fM and 800 fM for ricin demonstrating their extreme toxicity to eukaryotic cells. It has been shown [19] that abrin binds in greater amounts to Hela cells than ricin and that the time of onset of protein synthesis inhibition is dependent on the amount of toxin binding to the cells. In the present study it was found that the viability over time of BPE cells exposed to LC_{70} doses of abrin and ricin was virtually superimposable. This suggested that the mechanism of action of abrin and ricin are likely to be similar.

With BPE cells, a reduction in protein synthesis (measured by reduced ^{3}H-leucine incorporation) was apparent some 2 hours before a reduction in cell viability could be measured. Conversely, the decline in viability of rat intestinal epithelial cells closely paralleled inhibition of protein synthesis caused by ricin [20]. In addition a lag of thirty minutes between addition of ricin or abrin toxins and inhibition of protein synthesis in Ehrlich ascites cells has been noted [21]; this compares with our findings with ricin, although in the case of abrin, the onset of protein synthesis inhibition was after 90 minutes. Pulsing BPE cells with an LC_{70} or a 5 x LC_{70} dose of abrin or ricin indicated that at high doses of toxin, the period for effective intervention is likely to be short.

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